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Covid-19 Detection using qRT-PCR - A Review

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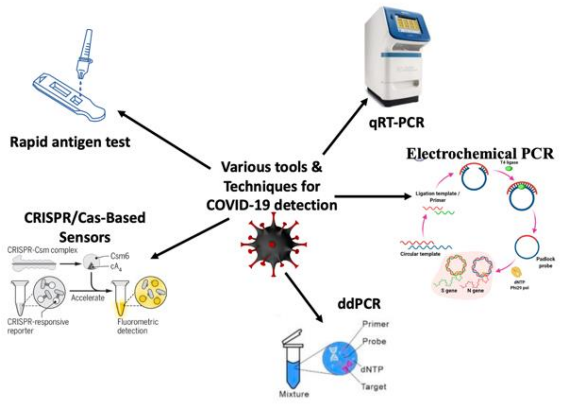
ABSTRACT

In the ongoing Covid pandemic, the monitoring of SARSnCoV-2 with the help of viral loads/viral kinetics has become more essential via the RT-PCR technique. However, the interpretations of qRT-PCR technique results are made as qualitative and quantitative or semi-quantitative, and day by day, this interpretation is becoming more important. Reverse transcription polymerase chain reaction is the most widely used technique for detecting viruses (rRT-PCR). Due to probable false-negative or false-positive findings, present techniques must be improved to avoid incorrect conclusions. Researchers have developed a multiplex rRT-PCR diagnostic method that simultaneously targets viral genes (RdRP and E) and one human gene (RP). The values of the Cycle threshold called Ct values that are a result of the RT-PCR test are highly affected by the variations attained among the different runs required to be operated and must be determined by the laboratories, especially in the quality control of quantitative RT-PCR. Somewhere, batch effects also play an important role in Ct value. Regrettably, several papers on Covid-19 used ingenuous values for Ct from qRT-PCR, which are the incorrect quantitative analysis unit. Qualitative analysis and Quantitative analysis both are of having different meanings; interpretation of Ct values cannot be interpreted directly as viral load; it must need a reference material with standard curves. The tractability and validity of the standard curve are the basis of the evaluation of the values. These factors help attain the accurate quantification of the expected number of viral copies.

Keywords- Coronavirus, Ct-value, detection, RT-PCR.

Graphical Abstract



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SUMMARY

Molecular diagnostics has been the front runner in the world's response to the COVID-19 pandemic. Particularly, reverse transcriptase-polymerase chain reaction (RT-PCR) and the quantitative variant (qRT-PCR) have been the gold standard for COVID-19 diagnosis. However, faster antigen tests and other point-of-care (POC) devices have also played a significant role in containing the spread of SARS-CoV-2 by facilitating mass screening and delivering results in less time. Appropriate application, quality control, and standardization must be addressed, and each stage of the experimental technique must be considered, from lab setup through sample capture and template preparation to RT and PCR. Only after validating each step can quantitative data be trusted. Chemicals, primers, probes, and equipment must be suitable for the quantification. Data interpretation remains a difficulty. Significant technical issues remain, notably with RNA-to-cDNA conversion and amplification. These issues suggest a new generation of RT-PCR tests. Presently being employed are the most recent developments in the creation of miniature PCR systems with a focus on COVID-19 detection. CRISPR/Cas technology's is potential for POC diagnostics. qRT-PCR assays, when carried out appropriately, are the method of choice for RNA detection and quantification.

I. INTRODUCTION

Coronavirus belongs to the Coronaviridae family showing symptoms of illness like the common cold, coughing, sneezing, shortening of breaths, kidney failure, and fever ranging from moderate to high, often called pneumonia in more severe case [1]. This deadliest disease originated as a new strain discovered in late 2019, transmitted from China. It has not been seen in humans before this. Our environment has altered dramatically since the first reports of the coronavirus disease 2019 (COVID-19) epidemic in December 2019. However, although some nations may be showing signs of recovery, others are still reporting a steady increase in cases [2]. Controlling the COVID-19 pandemic has relied heavily on the early detection of the disease by clinicians. SARS-CoV-2 RNA was originally detected in patient samples using molecular nucleic acid amplification techniques. With the ability to identify target nucleic acids (less than 100 copies per mL) with extraordinary sensitivity, RT-PCR and its quantitative variation have become keystones for diagnosis of SARS-CoV-2. An in-house laboratory could only manage this labor-intensive analysis, which took up to several hours to complete. Some RT-PCR tests have alarmingly high false-negative rates. Because of this, researchers began looking for quicker, less expensive, and more sensitive point-of-care (POC) biosensing devices that might be used for mass screening.

Reverse transcription and polymerase chain reaction (qRT-PCR) As a standard for detecting and quantifying RNA targets, [1] has gained widespread acceptance in the scientific community [2] and is now considered a standard research tool. qRT-PCR-based clinical diagnostic tests have also been developed as a result of its high-throughput potential, frequent release of better or innovative chemistries, more dependable apparatus, and improved procedures [4–6]. Even while qRT-PCR tests aren't always more sensitive than standard RT-PCR, they offer a number of advantages: It is possible to analyze samples with a target abundance that differs by orders of magnitude because of their wide dynamic range and low inter-assay variation; and In order to ensure repeatable findings, fluorescent reporter molecules are used to monitor the amplification products throughout each PCR cycle. This avoids the need for post-PCR analysis and allows for accurate data to be generated. This is critical because quantifiable data is needed in molecular medicine, for example, to measure viral load or evaluate response to treatment in haematological malignancies [7-9]. An RNA-dependent DNA polymerase is used to convert the first nucleotide of an RNA sample into DNA, which is then used as a template for further steps in RT-PCR (reverse transcriptase). Because of this extra step, the findings of the test are more brittle and unpredictable [10,11]. Although this approach has its drawbacks, the potential it holds led to a concerted effort to design diagnostic tests that take use of the assay's advantages while avoiding the assay's weaknesses.

II. STRUCTURE OF CORONAVIRUS

The structure of coronavirus is enveloped, non-segmented, surface projections like spikes, single-stranded resembling positive-sense RNA viruses whose genome is about 26-32 kilobases in length [2] (figure-1). The earlier studies showed that they cause zoonotic diseases, but later, they came under the category of communicable diseases [3]. The observation of coronavirus under an electronic microscope showed that it resembles the structure of Crown [4].

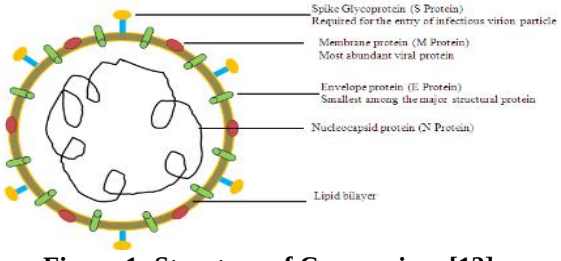

Figure 1: Structure of Coronavirus [12]

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To transmit respiratory infection, droplets of different sizes must have diameters larger than or equal to 10 meters, whereas droplet nuclei must have diameters below 5 meters [5]. COVID-19 virus is greatly spread through respiratory droplets through contact routes, as per current findings. COVID-19's transmission mechanism, the involvement of asymptomatic infected persons, the speed of its expansion, the possible interactions with wildlife or livestock, urban or rural areas, and population density are just some of the aspects that could affect its spread (figure-2). Microbes within droplet nuclei, which are commonly defined as particles 5 nm in diameter, can persist in the air for lengthy periods and can be communicated to others across distances larger than 1 m in airborne transmission [6,9]. The basic difference between SARS-CoV-2 and COVID-19 is explainig in (figure 3).

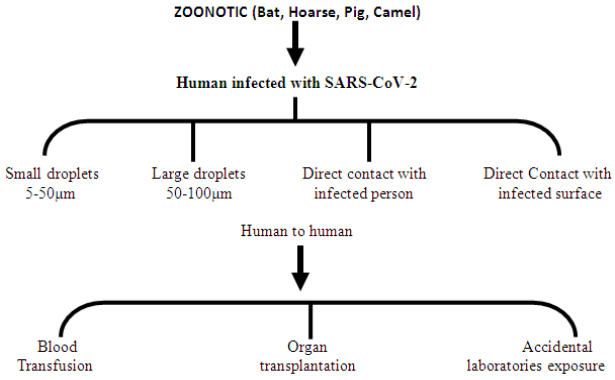

Figure 2: SARS-CoV-2 transmission routes [13]

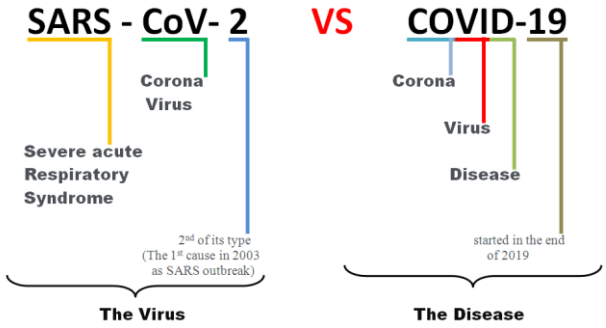

Figure 3: Clarity of SARS-CoV-2 & COVID-19

III. COVID-19 DIAGNOSIS ASSAY TECHNIQUES

Viral DNA Screening Using a Molecular Techniques

A number of COVID-19 clinical diagnostic tests have been adopted in the weeks following the entire genetic diversity of SARS-CoV-2 was uploaded to the Global Initiative on Sharing All Influenza Data (GISAID) website on January 10, 2020, by researchers and research organizations. The provision of sequencing data has aided in the progress of specific SARS-CoV-2 testing by allowing the creation of probes and primers [10].

Identification of SARS-CoV-2 Viral DNA using molecular techniques

RT-PCR, short for Reverse Transcription Polymerase Chain Reaction, depends on its capability for the amplification of a small amount of the virus genetic material encoded in the specimen. The RT-PCR technique is also termed the gold standard technique for the characterization and detection of the virus SARS-CoV-2 [11]. In the current scenario of the Covid pandemic, samples from the upper respiratory system are widely used. This study can also be done using Stool, ocular secretion, and serum based on the preferences. The Rutgers Clinical Genomics Laboratory recently designed an assay of RT-PCR as a TaqPath Covid-19 combo kit. This kit uses a method of saliva collection on its own. It is considered quicker and less pain-causing, contrary to other methods of sample collection. It lowers the risk of Covid attainment for those who provide healthcare facilities and enables high volume testing [15].

Molecular diagnostics relies heavily on real-time RT-PCR. This method for screening and early diagnosis of COVID-19 has been widely employed in the past. RT-PCR is particularly sensitive since it can produce and recognize a single copy of the specified genomic sequence. RT-PCT is also a quantitative technique because the amount of RNA copies synthesized in PCR grows significantly and is directly related to the amount of starting material, i.e., viral load. Early diagnosis of COVID-19 based on real-time RT-PCR assays is currently the most widely accessible commercial technology (test) [16]. Fig. 5 depicts the real-time RT-PCR diagnostic method in action. For the most part, real-time RT-PCR is employed to amplify SARS-CoV-2 genomic sequences (s). As a first step, viral RNA is isolated and purified from swabs taken from the nose or mouth and used for this purpose. Reverse transcriptase converts purified RNA into cDNA (complementary DNA). Amplification of the cDNA by PCR follows (Fig. 5)

Initiation, with the help of enzyme RNA Dependent DNA Polymerase, also called Reverse Transcriptase, the change of viral genome of RNA from DNA takes place. It is done by using a small sequence of DNA primers which are specifically designed for the recognition of complementary sequences onto the viral RNA genome, and the enzyme reverse transcriptase is used in the generation of copies of small complementary DNA, i.e., cDNA of the viral RNA.

Amplification, the monitoring of multiplication of DNA, is done in Real-Time with the progression of PCR. It uses a dye called fluorescent dye, or a DNA probe whose labelling is done using fluorescent molecule, and this probe is sequence-specific. In TaqMan assays, a quencher molecule can be used. Repetition in amplification by an automated system takes about 35-40 cycles until it detects the viral cDNA, most probably by a fluorescent dye or an electrical signal [17] (figure-4).

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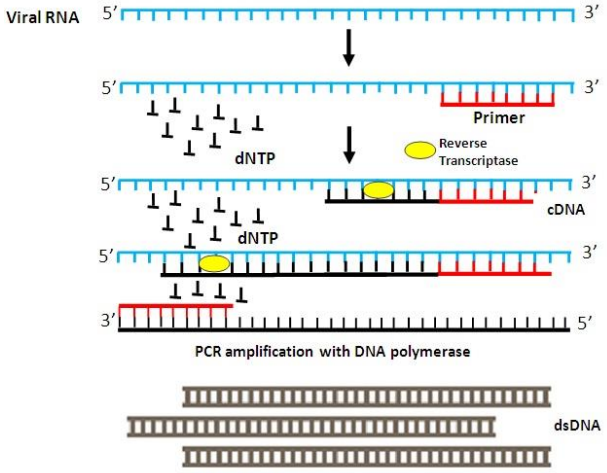

Figure 4: The RT-PCR produces a DNA copy of a particular section of the viral RNA transformed to dsDNA exponentially replicated. [18]

IV. RT-PCR BIOSENSORS

4.1. Digital RT-PCR

Vogelstein and Kinzler were the first to propose the idea of digital PCR (dPCR) in 1999 [19]. There are numerous sub-reactions in a dPCR reaction mixture, and the original numbers are calculated by counting those partitions that produce both positive or negative results (Figure 2). Without the need of any standards or control genes, as well as interference factors such particular template amplification inhibitors, this method is far more robust [20]. The Poisson's distribution is used to assess the quantification results in order to properly estimate small quantities of nucleic acid samples. In order to make an accurate SARS-CoV-2 diagnosis, it is required to use dPCR because of its high sensitivity, accuracy, and inhibitor resistance. Microfluidic digital polymerase chain reaction (dPCR) is one of the three ways of liquid separation that may be used for digital polymerase chain reaction (mdPCR). The detecting platform's sample partitioning technique is the key difference between these three types of digital PCR. A water-in-oil emulsion is used for the ddPCR test, whereas cdPCR employs an active partitioning method. This device consists of two microwell arrays on either half of the chip. The chambers are arranged in such a way that the two halves create a single channel [21]. Microfluidic chambers are used in mdPCR for sample splitting. The fluidic nature of these chambers allows for tens of thousands of wells to be created for each sample.

Anti-SARS-CoV-2 medications may be tested for efficacy, and viral mutations can be detected using PCR. It is possible to utilize PCR to measure a low viral load. In the case of COVID-19 testing by dPCR, samples from the blood, feces, sputum, and nasal and throat swabs may all be used. This method of SARS-CoV-2 detection has been shown to be more accurate and sensitive than the RT-PCR method [22]. According to Lu and colleagues, the detection limit of RT-dPCR is ten times lower than that of RT-PCR. Three hundred and eighty-eight samples from 36 COVID-19 patients were evaluated to compare the results of the real-time polymerase chain reaction (RT-dPCR) with the real-time PCR (RT-PCR). In the RT-dPCR, four pharyngeal samples that had been negative in the RT-PCR were positive. Another study found that patients who tested negative for RT-PCR had positive results for ddPCR [23]. The ddPCR results were validated by the presence of anti-COVID-19 antibodies in the serum. SARS-CoV-2 virus loads may be improved and more precisely quantified using the ddPCR [24]. RNA extraction and purification was used in the majority of published ddPCR techniques, which increases the risk of amplification mistakes [25]. There have also been direct measurements of the nucleocapsid (N) and envelope (E) genes by ddPCR, as well as the ORF1ab gene. Sputum, spit, nasal swabs, blood, and urine may all be used to measure viral load [26]. While using dPCR droplets to identify SARS-CoV-2 RNA in airborne aerosols, it was discovered that several medical staff and patients had a high viral load in the restrooms they frequented. The importance of sanitization and ventilation in reducing the spread of COVID-19 was shown in this investigation. High sensitivity and high throughput processing are essential advantages of dPCR for COVID-19 detection. The FDA has authorized the use of three commercial dPCR tests in the event of a medical emergency.

While dPCR may be utilized in regular diagnostics, there are some issues that need to be addressed first. DPCR is no different from regular PCR testing in that it likewise needs costly equipment and reagents, as well as a team of professionals to do it. The manufacturing of dPCR chips requires a number of sophisticated procedures, which makes the process expensive. In addition, as with other POC tests, accurate findings from dPCR equipment need adherence to certain standards and protocols [27].

4.2. Electrochemical PCR: Unexplored Potential

A working electrode is modified by biomolecules such that it may interact with specific analytes present in an aqueous electrolyte and produce an electrical signal that is proportionate to their concentration. It's possible to measure how much DNA has been amplified by electrochemical PCR by measuring an electroactive species' signal of either oxidation or reduction. A working electrode is modified by biomolecules such that it may interact with specific analytes present in an aqueous electrolyte and produce an electrical signal that is proportionate to their concentration. Electrochemical signals are generated and used to further quantify the tagged amplified products.

An electrochemical system may be easily integrated into small and sophisticated systems, allowing for great flexibility and real-time detection. As an added bonus, the long-term durability of electrochemically active labels makes them an important consideration for

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commercial electrochemical RT-PCR applications (EPCR). Electrochemical biosensors have lower power and sample volume requirements than RT-PCR. COVID-19 detection has elicited substantial attention from researchers, but the healthcare sector remains unwilling to deploy electrochemical biosensors for practical and commercial purposes.

It was during the pre-COVID period when electrochemical assays for detecting nucleic acid targets, such as cancer-related genes, emerged [28]. Many scientists believe that electrochemical tests can rival current PCR technology in terms of speed and sensitivity, while also eliminating the need for expensive reagents and dyes [29,30]. In the past five years, some research has been done on PCR-integrated electrochemical biosensors. PCR-free electrochemical sensors for SARS-CoV-2 RNA detection have been established in recent research with exceptional detection limits [31], although none have yet attained commercial or approved status [32]

The capacity of the sensing surface to endure the extreme temperature variations and salt concentrations necessary during PCR is a significant hurdle when integrating PCR with electrochemical transducers [33]. Isothermal amplification is preferable over PCR for use with electrochemical sensors because it does not need a temperature change. SARS-S CoV-2's and N genes might be detected using a rolling circle amplification (RCA)-based fast electrochemical detection technique (Figure 3). Comparative pulse voltammetry electrochemical detection was made possible by the use of sandwich hybridization in conjunction with oligonucleotide probes marked with redox-active. One copy of the N or S viral gene may be detected within two hours using this test with good selectivity and sensitivity.

4.3. CRISPR/Cas-Based Sensors: The New Alternative

"CRISPR" stands for clustered regularly interspaced short palindromic repeat, an antiviral technique that uses bacterial genetic information. Nucleic acid diagnostics benefit from the precision and specificity of CRISPR/Cas genome editing technologies. This kind of sensor relies on one guide RNA, which is coupled with the Cas system, to recognize target DNA and then provide an output signal. RT-PCR systems with a high level of specificity are an intriguing option. Assay specificity may be improved and turnaround times reduced using CRISPR/Cas-based diagnostics [34]. Recently, Hou et al. developed a quick test dubbed CRISPR–COVID for SARS-CoV-2 detection that has a turnaround time of less than 40 minutes compared to metagenomics sequencing and RT-PCR [35]. The elimination of RNA separation and amplification is another benefit of CRISPR/Cas systems, which makes this an analytical technique that is both quicker and more accurate. Using an ultra-sensitive RT-RPA CRISPR–fluorescence detection system for SARS-CoV-2 detection, RNA isolation may be avoided. In this method, the viral RNA is amplified using a cocktail of chemicals in saliva before being subjected to the CRISPR/Cas system for fluorescence signal amplification [36]. In keeping with the qRT-PCR results, the linear range of this portable CRISPR test was determined to be 1 to 105 copies/mL with a detection limit of 0.38 copies/mL. With CRISPR-Cas13a, the detection of SARS-CoV-2 RNA from swabs has been improved by eliminating pre-amplification [37]. The most significant component of this study was the use of several sets of crRNAs to activate a greater number of Cas13a per target RNA. A CRISPR-based assay for COVID detection was also shown to be very sensitive compared to existing CRISPR-based assays, as demonstrated by the capacity to directly convert the fluorescent signal into viral loads.

V. qRT-PCR : QUANTITATIVE APPROACH

qRT-PCR assays work on the simple premise that when RNA is transcribed into cDNA, a detection chemical, an equipment, and software are all required to determine whether or not PCR products have been produced [38]. An rise in fluorescence occurs more quickly when the nucleic acid target has a greater beginning copy number. There are two types of detection chemistries: those that use probes and those that don't. SYBR Green I binding to dsDNA is the most often used non-probe-based chemical [39]. Unbound dye fluoresces in solution, but the nascent double-stranded DNA binds more and more of it throughout the PCR experiment. As the polymerization process progresses, the fluorescence signal increases while it is being tracked in real time [40] A melting curve of the amplicon may be generated by graphing fluorescence vs. temperature (Figure 2). Non-probe-based chemistries have the benefit of being able to quickly transform improved traditional RT-PCR experiments into real-time assays [41]. Because they are still reliant on the primer specificity, this is a significant drawback. Probe-based chemistries use fluorescent amplicons that only glow when they hybridize with the complementary target of their corresponding amplicon (Figure 3). Due to this extra specificity provided by probe-based chemistries, formerly independent validation steps are now integrated into the RT-PCR process. Multiple targets, such as infectious pathogens or biomarkers, may be detected and differentiated using multiplexing in a single tube. Non-probe-based chemistry has recently been created that promises to make multiplexing easier than other non-specific chemistries [44]. iG and iC, which can only couple with each other, are used in this method. A single iso-dC at each of the five ends of the downstream (sense) PCR primers is tagged with a variety of fluorophores. Fluorescence quenching during amplification is increased by the addition of a quencher labeled iso-dG to the amplification master mix [24]. As 'Plexor,' Promega

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offers this technology. Using probe-based chemistry, various fluorophores are used to label each probe. A non-specific interaction between primers and probes may occur because of the three oligonucleotides per target. Careful assay design and reagent selection are thus required [25]. However, quadruplex (quintuplex on the ABI 7500) tests are now conceivable, although they are beyond the capability of the technology. As chemistries continue to improve and validate, multiplex qRT-PCR will play a major role in clinical diagnostic tests [20, 21] An alternative is to use two different sets of primers in two different reactions to prime each of the amplification reactions. The first reaction uses two primers from outside the reaction, and the second reaction uses two internal primers and a hybridization probe from inside the second reaction. This is known as a "nested qPCR" assay [28]. The SARS virus was detected early using nested PCR tests, which have been shown to increase analytical sensitivity [29,30]. There is an ever-present issue of increasing contamination susceptibility, and investigations including a greater number of clinical specimens are needed to determine the real sensitivity and specificity of this approach.

VI. QUANTIFICATION STRATEGIES

For quantitative assessments, one or more co-amplified control mRNAs may be supplied [2]. An external standard may be employed to construct Ct (threshold cycle) standard curve by serial dilution against a target copy number [45] first. These two y-intercepts, y-intercept (sensitivey) and y-slope (amplification efficiency), may be utilized to calculate the copy numbers of unknown samples using linear regression. It is possible to construct standard curves using many methods including commercially available universal reference RNAs, in vitro T7-transcribed sense RNAs, PCR fragments, and single-stranded sense-strand synthetic oligodeoxyribonucleotides. Viral or tumor load in bodily fluids may be quantified using this approach. Absolute quantification relies only on precision of the criteria to be used. Standard curves, in general, enable the development of specified and consistent outcomes and are highly reproducible. External standards, on the other hand, are unable to identify or adjust for the presence of inhibitors in the samples. One or more internal reference genes may be used for comparison, and the Ct values from the target RNAs can be used as a target-specific Ct ratio. There must be a close match between the amplification efficiency of target and reference genes in order to ensure the correctness of any expression result that is computed For a more accurate calculation of the actual expression ratio, a number of mathematical models have been developed that use various efficiency-correcting methods [46, 47]. Relative quantification may be deceiving since the expression of most reference genes is regulated and their levels typically shift drastically with treatment or between individuals. For this reason, it's possible that, in certain cases, when a Ct value for a target RNA is first seen, the relative amounts of reference and target RNA have already reached plateaus of many orders of magnitude. If this isn't taken care of, it might affect the accuracy of RNA measurement.

VII. DETECTION OF POSITIVE-STRAND RNA VIRUSES

In the genomes of positive-stranded RNA viruses, the RNA polymerase is unique to the virus and is built up of ss'sense' RNA. For example, the antisense replication intermediates created by the RNA are used to generate a high number of complementary DNA molecules (cDNA). Viruses such as enteroviruses, rhinoviruses, and coronaviruses are included in this group as well. qRT-PCR assays came in handy after the SARS outbreak. By November 2002, SARS had spread to 29 countries, infecting over 8,000 people and claiming 774 lives. To accurately identify the SARS virus during the initial few days of sickness, RT-PCR assays were found to be insufficiently sensitive, according to researchers. SARS epidemics necessitated a huge volume of specimens to be examined, and the assay's high throughput capability was important. SARS coronavirus loads and rates of positive in patients' upper respiratory tracts peaked about day 10 following the commencement of the sickness, unlike previous respiratory viral infections, which peaked at around day 7 after the onset of the illness [48]. Lower respiratory tract specimens had the greatest viral load, and nasopharyngeal aspirate had a greater viral load than throat swabs [49]. In week one, fecal samples had a high viral load and were chosen as the specimen of choice for week two. Prognostic information may be gained by using the qRT-PCR test. Patients who had diarrhea and were admitted to the intensive care unit had significant virus loads in their nasopharyngeal aspirates, which were used to predict mortality [46]. qRT-PCR testing may now be run on a regular basis to rule out the existence of other respiratory viruses in order to rule out SARS-associated coronavirus [50]. These viruses may also be detected using quantitative real time polymerase chain reaction (qRTPCR). When it comes to the clinical diagnosis of viral meningitis, qRT-PCR is substantially more sensitive than viral culture for the identification of enterovirus in CSF fluid [51, 52]. Additionally, a test is available that targets norovirus, a frequent causative agent of acute gastroenteritis. When using the identical primer sets, this assay is 4 orders of magnitude more sensitive than the standard design, and it's on par with an equivalently sensitive conventional RTPCR [53].

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VIII. RT-PCR-ASSOCIATED PROBLEMS

For disease-related indicators, there are a number of factors that might lead to unclear results [11].

8.1. RNA purification

RNA may be extracted from a variety of clinical samples, including blood and other physiological fluids, as well as solid biopsies obtained during endoscopy, surgery, post-mortem, and from archives. Naked RNA is quickly degraded by endogenous RNases (ribonucleases), which are present in every living cell. It is vital to ensure that no endogenous or exogenous RNases are introduced during extraction in order to effectively separate high-quality RNA and to accurately and relevantly compare the findings of qRT-PCR [54].

Recent studies have shown that qRT-PCR tests may effectively quantify RNA isolated from FFPE archival materials. Samples that are fewer than 10 years old may be amplified by RT-PCR and the mRNA expression levels can be determined. Molecular discoveries may be linked to patient response to therapy and final clinical outcome in research like this, which is critical. Samples with an average nucleotide length of 200–250 nucleotides are excellent for qRT-PCR tests, which have amplicon lengths of less than 100 bp. The gene expression profiles derived from FFPE samples (r2 = 0.69) do not totally match those generated from the similar frozen samples. The same authors note that just 38 genes were found to be differently expressed in the equivalent FFPE samples, despite the fact that 64 genes were shown to be so in the matched fresh-frozen normal colon and cancer samples. Any findings based on FFPE samples must be validated by independent experimentation, but may potentially overestimate or misreport gene expression patterns [55].

8.2. cDNA synthesis

The RNA template may be utilized as a template for cDNA synthesis using random primers, oligo(dT), a combination of the two, or target-specific primers. The discrepancies in primer selection make it impossible to compare the results produced using different methodologies [56]. cDNA synthesis may be successfully primed without the insertion of any primers, which is also unknown [57].

8.3. Random primers

There are numerous cDNA transcripts for each original target when using this approach, which is why it generates the most cDNA. RNA-derived ribosomal DNA might even compete with an extremely low concentration of a target for the bulk of the transcripts created by whole-RNA extraction. A pre-incubation at a lower temperature is essential since the Tm of random primers is so low, if they are to be used with thermostable RT enzymes. This is by far the worst way to make cDNA [58].

8.4. oligo(dT)

It is more specific than random primers to synthesize cDNA using oligo(dT), since this will not result in priming from rRNA. Although it will not prime any RNAs without a polyA+ tail, this is the ideal approach to utilize when trying to produce an accurate cDNA copy of the mRNA pool. Due to secondary structures or an unusually lengthy primer/probe-binding site, the primer/probe-binding site may not be accessible to the RT (mRNA). In certain cases, oligo(dT) may be combined with random primers; however, this might lead to errors in oligo(dT) measurement (dT) [59].

8.5. Inhibitors

For reliable mRNA quantification, the presence of RT-PCR inhibitors during template preparation might be a major hindrance [60]. Haemoglobin, urea, organic and phenolic chemicals, and lipids are all examples of common inhibitors in clinical and forensic research [61]. Remaining heparin, DNA fragmentation and proteinase K-digested hemoglobin are all factors that might affect the effectiveness of PCR. Because different patients' amplification efficiencies and hence Ct values for the same target are affected by this kind of inhibitor, it is difficult to compare quantitative Real Time-PCR results from several affected role or samples from the same individual. It's worrying that PCR inhibitors have been identified in laboratory plasticware [62]. Lot-to-lot variance is an important factor to consider when evaluating test reproducibility with reagents. For example, the susceptibility of polymerases to inhibitors like blood or ions might vary greatly [63]. The use of a suitable thermostable DNA polymerase may thereby reduce or eliminate the PCR-inhibiting impact of different components in biological sample.

IX. IMPROVING THE SENSITIVITY OF THE RT-qPCR ASSAY

Primer concentrations, degenerate primers, and multi-target detection are the three primary strategies to increase RT-sensitivity qPCR's from a primer standpoint. Premer concentrations for probe-based tests ranged from 30 to 90 nanometers [64]. RT-sensitivity qPCR's may be improved by increasing the concentration of primers suitably. The primer concentrations in all experiments were within this range (data not shown). Vijgen et al., for example, discovered that increasing the primer concentration per reaction up to 400 nm may enhance the assay's sensitivity [54]. SARS-CoV-2 has a wide range of genetic variants, hence degenerate primers are utilized to counteract this. A total of three tests, including the E gene assay and the RdRp assay and the N gene assay, were employed to identify pan-sarbecovirus.

When evaluating primers and probes, consider their sensitivity as well as their specificity. Evaluation of specificity involves looking at whether there is cross-reactivity, which is critical. Neither a lack of sensitivity

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nor limits in viral detection technology have led to an underestimation of co-infections [57]. For this reason, regardless of the presence of another respiratory pathogen, all patients who fulfill the suspected case criteria should be tested for the presence of COVID-19.

X. DISCUSSION

The global growth of COVID-19 necessitates the development of viral detection methods that are faster, more accurate, and more sensitive [65]. In order to create a reliable, efficient, and sensitive detection method for the SARS-CoV-2, significant research is ongoing. This study involves developing an assay that simultaneously detects both the viral RdRP or E gene and one human RP gene in a single reaction tube. It's COV2-kit that's being tested here. Probes with separate fluorescent dyes may be used in the same reaction tube of the Applied Biosystems 7500 Fast Real-Time PCR System to detect gene amplification (HEX, ROX, and FAM). The procedure was optimized using three distinct approaches: basic (targets only one gene), duplex (targets 2 genes concurrently), &triplex (at the same time targets 3 genes) [66]. For this, a synthetic viral template and RP gene mRNA were utilized along with a viral template. The response Ct values vary from 24 to 34. To put it another way, a sample must have a Ct value less than 37 to be considered positive.

All examined methods' Ct values fell below this limit (simplex, duplex, or triplex). RdRP and E genes have LODs of at least 10 copies per L, respectively. The RdRP probe and primers were more sensitive than the E primers and probe. Test effectiveness is highly dependent on the COVID-19 patients' viral load. It's safe to say that the RdRP gene has the best sensitivity for detecting low viral loads (10 copy/L). The E gene may be used as a target to identify patients with a viral load less than or equal to 10 copies per litre. These two sensitive gene targeting methods can thus identify samples with a low virus burden. The reaction also contains a human gene target, RP, as an internal control in addition to the viral genes. Each response is examined using a separate tube, which reduces the number of samples that can be analyzed and raises the cost. The number of responses per sample may be cut in half using the present approach [67]. Thus, evaluating patients in 96-well plates each run takes lower time and costs less money because of the assay. Designing and utilizing efficient primer/probe sets allows for rapid, high-sensitivity, reliable and low-cost detection of SARS-CoV-2.

XI. CONCLUSION

With this review paper, we can conclude that RT-PCR is a kind of technique that is helpful in the detection of COVID strains in the host body, and it is helpful in the illustration that it is present in what stage of infection. SARS-CoV-2 was diagnosed using a multiplex rRT-PCR technique, which was shown in this research. There are 2 viral genes, i.e., RdRP and E or one human gene, i.e., RP gene, which is simultaneously targeted in the similar PCR process to offer a precise, dependable, and simple-to-use SARS-CoV-2diagnostic method. This is a breakthrough. A further benefit is that it may be used on many patients while utilizing a less amount of PCR reagent. Because of this, the test's price and accuracy have both increased. Because of this, it is important to test the primer/probe sequences against newly discovered mutants. The test has to be verified with various RT-PCR equipment and may be utilized in diagnostic labs, medical facilities, hospitals, and infection control organizations.

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Declaration of interest statement

- ***No conflict of interest exists***

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